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Immunology

Mouse stem cell antigen Sca-2 is a member of the Ly-6 family of cell surface proteins

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ABSTRACT **Mature T lymphocytes arise from intrathymic T-cell precursors, which in turn are derived from a multipotent stem cell in the bone marrow. Unlike bone marrow stem cells, the differentiation potential of the earliest intrathymic precursor cells is strongly biased toward the lymphoid lineage. The major difference in cell surface phenotype between early thymic precursor cells and bone marrow stem cells is that the former population expresses Sca-2. The progeny of the intrathymic precursor population continue to express Sca-2 until the transition from blast cells to small cells, at which stage expression of Sca-2 is down regulated. Mature thymocytes and peripheral T cells do not express detectable levels of Sca-2, whereas peripheral B cells are Sca-2-positive. We report herein the complete sequence of mouse Sca-2 deduced from a thymocyte cDNA clone. Sca-2 is a member of the Ly-6 family, a group of small cysteine-rich cell surface proteins that are anchored in the membrane by a glycosyl-phosphatidylinositol moiety.**

T lymphocytes develop from a multipotent stem cell that seeds the thymus from fetal liver and adult bone marrow (1, 2). A bone marrow stem cell population with the potential to reconstitute multiple hemopoietic lineages is also able to colonize an irradiated thymus with high efficiency after intrathymic transfer (3, 4). The earliest intrathymic precursor cells from adult thymus are found within a small population making up 0.05% of total thymocytes (5). Intrathymic transfer of this purified precursor population into irradiated recipients gives rise to T lymphocytes of the $\alpha\beta$ and $\gamma\delta$ lineages, with repopulation kinetics similar to that of thymus-seeding bone marrow progenitors (5). Since the intrathymic precursors have lost the ability to differentiate along the erythroid and myeloid lineage (6), they are the earliest known intrathymic lymphoid committed precursor. The cell surface phenotype of the intrathymic precursors is similar to that of bone marrow stem cells, except that the former express Sca-2 (6). Sca-2 is also expressed by the majority of immature thymocytes, but not by mature peripheral T cells. Sca-2 is also expressed on B220$^+$ bone marrow cells and peripheral B cells, including germinal center cells (7).

We report the complete primary structure of the Sca-2 molecule deduced from a cDNA clone isolated by expression cloning.† The Sca-2 molecule is a small Cys-rich protein anchored in the cell membrane via a glycosyl-phosphatidylinositol (GPI) moiety. Sca-2 is a member of the Ly-6 family, a group of molecules differentially expressed in several hematopoietic lineages that appears to function in signal transduction and cell activation.

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs). The Sca-2 mAb E3 81-2.4 (8) was obtained from G. Spangrude (Laboratory of Persistent Viral Diseases, Hamilton, MT). Other mAbs used in this study were anti-Thy-1.2, clone 30H12 (9); anti-CD4, clone GK-1.5 (10); anti-CD3, clone KT3-1.1 (11); anti-intercellular adhesion molecule 1 (ICAM-1), clone 1.H4 (12); and anti-CD8, clone D9 (13).

Immunofluorescent Staining and Flow Cytometry Analysis. Two-stage labeling of cell surface molecules was carried out using appropriate mAb supernatant at a saturating dilution, followed after washing by incubation with affinity-purified F(ab')2 goat anti-rat IgG conjugated with fluorescein isothiocyanate (FITC) (Caltag, South San Francisco, CA) (7 μg/ml) or FITC-conjugated sheep anti-mouse IgG (Silenus, Hawthorn, Victoria, Australia) (7 μg/ml). Propidium iodide (1 μg/ml) was included in the final wash. Cell surface fluorescence was assessed by FACScan analysis (Becton Dickinson). Forward light scatter and propidium iodide staining gates were set to exclude dead cells.

Expression Cloning and Characterization. Sca-2 cDNA clones were isolated from a thymus cDNA library by using a COS-cell expression system (14, 15) and immunoselection with anti-Sca-2 mAb and sheep anti-rat immunoglobulin Dynabeads (Dynal, Oslo). Two rounds of DEAE/dextran transfection and selection were performed to isolate positive clones. Restriction enzyme fragments were subcloned into M13mp10 and M13mp18. Nucleotide sequencing was by the dideoxynucleotide chain-termination method and each nucleotide was determined in both directions with Sequenase dGTP and dITP reagents (United States Biochemical).

Protein Sequence Comparisons. Sequence comparisons were made with protein sequences obtained from the Protein Sequence Database of the Protein Identification Resource (Release 37.0, June 30, 1993), National Biomedical Research Foundation (Washington, DC) under the following accession numbers: RWHU59, human CD59; RWBEM3, herpesvirus CD59 homolog; A25708, mouse Ly-6E.1; A46528, ThB. Other sequences were translated from the following nucleotide sequences from National Center for Biotechnology Information–GenBank; MUSLY6C2A, mouse Ly-6C; MMLY6F112, mouse Ly-6F; MMLY6G113, mouse Ly-6G; RATLY6B; rat Ly-6B; RATLY6CA, rat Ly-6C. The squid glycoprotein (Sgp) 2 sequence was from ref. 16. The ALIGN program was used to determine statistical significance of sequence similarities (17), using the mutation data matrix with a matrix bias of +6, a gap penalty of 6, and 100 random runs.

Cleavage of GPI-Linked Cell Surface Molecules. Unfractionated thymocytes (2.5×10^6 cells) from female C57BL/6 mice were incubated with 190×10^{-3} units of phosphatidylinositol-specific phospholipase C (PI-PLC, Boehringer Mannheim) in 50 μl of Hepes-buffered mouse tonicity balanced salt solution (150 mM NaCl/3.7 mM KCl/2.5

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Abbreviations: Sca, stem cell antigen; mAb, monoclonal antibody; GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; ICAM-1, intercellular adhesion molecule 1; Sgp, squid glycoprotein; uPAR, urokinase-type plasminogen activator receptor.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U04268).

mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM K_2HPO_4/0.7 mM KH_2PO_4/15 mM Hepes, pH 7.2) at 37°C for 90 min. Cells were washed and then labeled with individual mAbs, followed by F(ab')2 goat anti-rat IgG conjugated with FITC. COS cells that had been transfected 2 days earlier with either Sca-2.1 plasmid or ICAM-1/CDM8 plasmid were detached from culture flasks with EDTA without trypsin and treated with PI-PLC as above, except that 6×10^5 cells were used per digest.

RESULTS

Isolation of Sca-2 cDNA Clones. Sca-2 cDNA clones were isolated using the COS-cell expression cloning protocol developed by Aruffo and Seed (14). A C57BL/6 thymocyte library in the πH3M vector (kindly provided by B. Seed, Massachusetts General Hospital, Boston) was introduced into COS-M6 cells by DEAE-dextran transfection. After 2 days in culture, cells were detached with EDTA, washed, and selected with the anti-Sca-2 mAb (E3 81-2.4) and paramagnetic Dynabeads coated with sheep anti-rat immunoglobulin (15). Episomal plasmid DNA was recovered from the adherent COS cells and used to transform *Escherichia coli* by electroporation. Plasmid DNA was purified from the resulting colonies and a second round of selection was performed, after which 98 colonies were individually picked and grown overnight in 1.5-ml cultures. After pooling the cultures in groups of seven, plasmid DNA was purified and transfected into COS cells. After two rounds of transfection and immunoselection, five out of eight pools were positive for Sca-2 expression by flow cytometry (data not shown). The seven individual plasmids making up one positive pool were transfected separately into COS cells and two out of seven gave Sca-2 expression (Fig. 1). The level of Sca-2 expression was heterogeneous with only a proportion of the cells clearly positive. However, this resembled the level of ICAM-1 expression after transfection with the control plasmid ICAM-1/CDM8, and both were clearly positive compared to the mock transfection (Fig. 1). A low level of cell surface expression was also seen for a murine HSA (CD24) clone isolated from the same library (unpublished data). Thus, despite the relatively low levels of expression, this protocol is suitable for the isolation of cDNA clones encoding cell surface antigens.

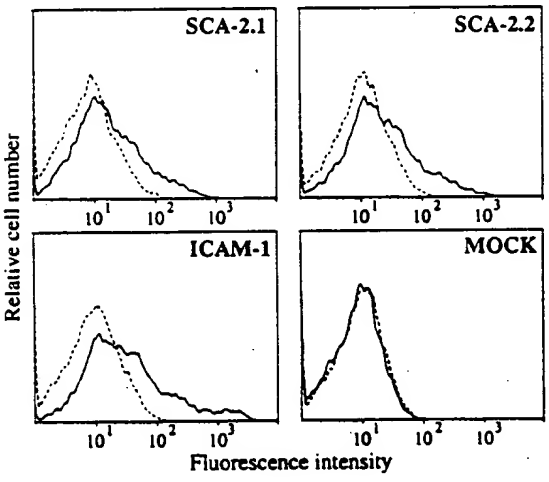

FIG. 1. Transient expression of Sca-2 on COS cells. Cells were transfected with the Sca-2.1 plasmid clone (*Upper Left*), the Sca-2.2 clone (*Upper Right*), or ICAM-1/CDM8 (*Lower Left*) or mock transfected (*Lower Right*) by the DEAE-dextran method. After 2 days, cells were detached and labeled with the anti-Sca-2 mAb (for Sca-2.1, Sca-2.2, and mock transfections, solid line) or with 1.H4 (anti-ICAM, solid line) for the ICAM transfection. The second-stage reagents were FITC-conjugated goat anti-rat IgG F(ab')2 for the Sca-2.1, Sca-2.2, and mock transfections and FITC-conjugated sheep anti-mouse IgG (Silenus) for the ICAM-1 transfection. Dotted lines represent staining by the following isotype-matched negative controls: KT3 for Sca-2.1, Sca-2.2, and mock transfections and D9 for the ICAM-1 transfection. Data from 10^4 cells are shown for each histogram.

cDNA Sequence Analysis. Both positive plasmid clones contained cDNA inserts of ≈1.1 kb and had identical restriction maps (data not shown). One clone (Sca-2.1) was sequenced and the complete nucleotide sequence of 1072 bp is shown in Fig. 2*A*. The cDNA sequence contains two potential translational start sites (ATG) in the same reading frame at positions 44 and 62. The sequence after the first ATG codon shows an open reading frame coding for 136 amino acids (Fig. 2*B*). The 3′ untranslated region contains a consensus polyadenylylation site (AATAAA) 14 nucleotides from a poly(A) tail of 14 nucleotides [poly(A) tail not shown in Fig. 2*A*]. The translated sequence immediately downstream from initiator methionine codon at position 44 shows a high proportion of hydrophobic amino acid residues, characteristic of a posttranslationally cleaved leader sequence. The most likely cleavage point for this sequence is after the Ser residue at position −1 (Fig. 2*B*). There is a stretch of hydrophobic amino acid residues at the C terminus of the protein that is a signal sequence for attachment of a GPI anchor (see below).

The mature Sca-2 protein contains 82 amino acids with a predicted molecular mass of 8.8 kDa. This sequence contains a relatively high proportion of Cys residues (10/82), which suggests that numerous disulfide bonds stabilize the tertiary structure of Sca-2. There is one consensus sequence for N-linked oligosaccharide attachment (Asn-79). Ser-51 and Ser-56 are both flanked by Pro residues and may be O-glycosylated (19, 20).

Sequence Homology Between Sca-2 and the Ly-6 Family. A computer-assisted homology search of the protein database with the Sca-2 sequence revealed significant homology to members of the Ly-6 family (Fig. 3*A*). In the mouse, the Ly-6 family is encoded within a cluster of at least 18 genes on chromosome 15 (21), which includes ThB (22). Sca-2 shows significant structural similarities to mouse Ly-6C, Ly-6E.1, Ly-6F, Ly-6G, and ThB; rat Ly-6B and Ly-6C; human CD59; and a herpesvirus CD59 homolog. The level of amino acid identity between Sca-2 and other members of the Ly-6 family is relatively low. However, a remarkable conservation of all extracellular cysteine residues is evident, with all 10 Cys residues in the putative mature protein conserved between Sca-2 and each sequence shown in Fig. 3*A* (except Sgp-2, where 8 of 10 Cys residues are conserved). The statistical significance of the Sca-2 sequence alignments with members of the Ly-6 family was assessed using the ALIGN program. The optimal alignment between two sequences is computed and expressed as the number of SD units this alignment score is displaced from the mean optimal scores for the same sequences randomized. An alignment score of ≥3 SD is usually regarded as statistically significant, particularly if consistent scores are obtained with a number of sequences from the same family (17). The best alignment score for Sca-2 and a member of the Ly-6 family was with Ly-6G (8.5 SD), a recently defined molecule expressed in bone marrow (23). Other alignment scores shown in Fig. 3*B* clearly support an evolutionary relationship between Sca-2 and the Ly-6 family. The lowest alignment score between Sca-2 and any member of the Ly-6 family was for Sgp-2 (4.0 SD). Sgp-2 has been shown to be distantly related to the Ly-6 family (16) and the low score obtained for Sca-2 with Sgp-2 is consistent with the phylogenetic distance between mammals and invertebrates.

Sca-2 Is Attached to the Cell Membrane by a GPI Anchor. The C terminus of the Sca-2 protein is enriched for hydro-

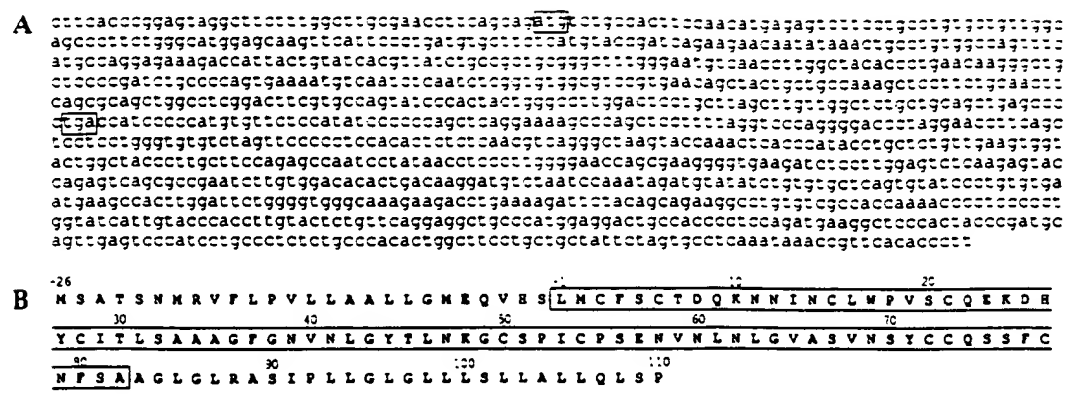

FIG. 2. (*A*) Nucleotide sequence of the thymocyte cDNA clone encoding mouse Sca-2. The methionine initiation codon and the stop codon are boxed. (*B*) Deduced amino acid sequence of Sca-2. The predicted sequence of the mature cell surface protein is boxed. The N-terminal amino acid, Leu-1, was assigned on the basis of homology to the Ly-6 sequences, and the C-terminal amino acid, Ala-82, was assigned according to GPI-attachment consensus sequences (18).

phobic amino acids, indicating that it is probably a signal peptide for the attachment of a GPI moiety for cell membrane anchoring (18). The overall sequence homology to the Ly-6 family supports this possibility, since all sequences shown in Fig. 3 have C-terminal hydrophobic signal sequences for GPI attachment or have a GPI anchor. The ability of PI-PLC to specifically cleave Sca-2 molecules from the surface of thymocytes and transfected COS cells was investigated. When thymocytes were treated with PI-PLC, a decrease in the mean cell surface expression of Sca-2 was seen, compared to the control sample in which cells were incubated for the same period without enzyme (Fig. 4*A*). In parallel samples, levels of a known GPI-anchored molecule (Thy-1) were also diminished, whereas the level of a non-GPI-anchored molecule (CD4) was unaffected (Fig. 4*A*). Sca-2 and Thy-1 vary in their susceptibility to cleavage by PI-PLC, which may reflect inherent structural differences between the two proteins or possibly steric constraints imposed by interactions with other unknown membrane molecules. The results of Fig. 4*B* show the specific cleavage of Sca-2 from the surface of Sca-2.1-transfected COS cells, in contrast to a parallel ICAM-1-transfected sample (a non-GPI-anchored molecule), which

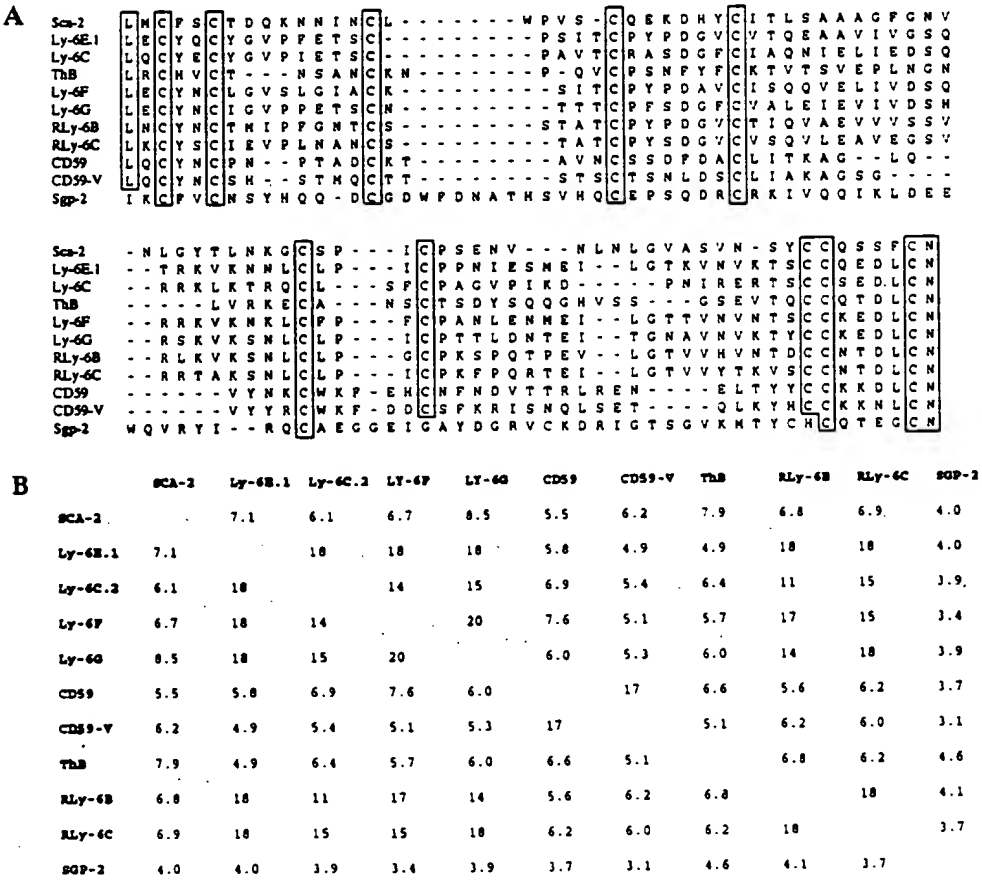

FIG. 3. (*A*) Sca-2 is related to the Ly-6 family of cell surface molecules. Alignment of the extracellular region of Sca-2 (residues 1–79) with the corresponding region of related sequences are shown. N-terminal and C-terminal leader sequences have been omitted for clarity. Residues common to Sca-2 and at least nine other sequences are shown boxed. (*B*) Alignment scores. The matrix of values shows the ALIGN scores in SD units for each of the sequence comparisons. The Dayhoff mutation data matrix was used with a matrix bias of +6 and a gap penalty of 6. The SD scores correspond to the following probabilities: 10^{-3}, 3.1 SD; 10^{-5}, 4.3 SD; 10^{-7}, 5.2 SD; 10^{-9}, 6.0 SD; 10^{-15}, 7.9 SD (17).

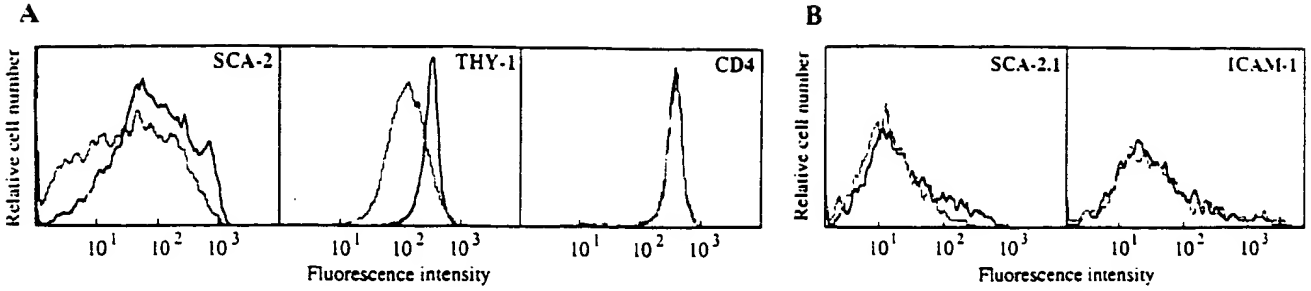

FIG. 4. Sca-2 is attached to the cell membrane by a GPI anchor. (*A*) Thymocytes were incubated at 37°C in the presence (vertical hatched lines) or absence (solid lines) of PI-PLC. Cells were labeled with the mAbs indicated then with FITC-conjugated anti-IgG as described in Fig. 1. The negative staining profile obtained with an isotype-matched negative control mAb is shown (stippled line). Data from 10^4 cells are shown for each histogram. (*B*) Sca-2.1-transfected COS cells (*Left*) or ICAM/CDM8-transfected COS cells (*Right*) were incubated in the presence or absence of PI-PLC and stained as above. Data from 3×10^3 cells are shown for each histogram.

was unaffected by PI-PLC. These results confirm that the predicted GPI signal sequence (Fig. 2*B*) directs attachment of a GPI anchor to the C terminus of Sca-2 and that this modification occurs in both thymocytes and COS cells. The conservation of the Cys-Asn sequence near the C terminus of the mature protein in all members of the Ly-6 family (Fig. 3*A*) suggests a consensus attachment point for the GPI anchor, since the corresponding Asn residue in Sgp-2 has been empirically determined as the C-terminal amino acid (16). However, data for known GPI attachment sites suggests that Ala-82 is the C-terminal amino acid of Sca-2 (18).

DISCUSSION

The Sca-2 antigen is a small Cys-rich cell surface protein of 82 amino acids and is a member of the Ly-6 family. Sca-2 is anchored in the cell membrane by a C-terminal GPI moiety, a posttranslational modification in common with each member of the Ly-6 family described to date. Several GPI-linked molecules have been implicated in signal transduction in hematopoietic cells, suggesting an important role for the GPI anchor in cell activation (24). For Ly-6A/E, mAb ligation induced a mitogenic signal to T cells in the presence of accessory cells or interleukin 1 (25, 26). Signal transduction in T cells via GPI-anchored proteins involves interleukin 2 production and this is dependent on the presence of a full-length T-cell receptor ζ chain (27). However, one report has shown that Ly-6A/E mAbs can induce a down regulation of an interleukin 2 response (28). It is possible that Sca-2 may also function as a signal transduction molecule, and the presence of a GPI anchor suggests a signaling mechanism in common with other GPI-anchored molecules.

Sca-2 is also related to human CD59, an Ly-6-related protein of broad tissue distribution that inhibits complement-mediated cytolysis of erythrocytes and leukocytes by competing with C9 incorporation into the membrane attack complex (30, 31). CD59 has been proposed as a second cell surface ligand for the CD2 antigen (32), although a recent report (33) shows that a multivalent form of soluble CD2 does not bind CD59. Another member of the Ly-6 family (not shown in Fig. 3) is the urokinase-type plasminogen activator receptor (uPAR), a major cell surface regulator of plasminogen activation (34). The uPAR sequence reveals three Cys-rich Ly-6-like domains compared to the single domain found in all other Ly-6 family members (35–37). The ligand binding activity of uPAR is contained within the N-terminal Ly-6-like domain, which binds an epidermal growth factor-like domain in urokinase-type plasminogen activator (35, 38). A sequence comparison of Sca-2 with the N-terminal domain of uPAR gave an ALIGN score of 4.1 SD (data not shown) showing weak but significant homology. Thus, it is conceivable that Sca-2 and other members of the Ly-6 family also bind ligands with epidermal growth factor-like domains. A recent report has shown structural homology between the Ly-6 family and several snake toxins (23), which suggests an important structural role for this domain in proteins of diverse function.

The Sca-2 antigen is expressed on immature thymocytes but not on mature thymocytes or peripheral T cells. Sca-2 is not confined to the T lineage, since B-lineage cells in the bone marrow and the periphery are also Sca-2$^+$. Nevertheless, within the T lineage, upregulation of Sca-2 expression appears to coincide with the transition from a multipotential bone marrow stem cell to an intrathymic lymphoid committed precursor cell (6). Further characterization of this lymphoid precursor is central to the understanding of early T-cell differentiation and lymphoid lineage commitment. The expression of Sca-2 by a lymphoid precursor cell upon entry into the thymus suggests an important function for Sca-2 in early thymopoiesis.

Note. While this manuscript was under review, the sequence of a mouse thymocyte antigen, TSA-1, was reported (29). The deduced protein sequence of TSA-1 is identical to the Sca-2 sequence reported herein, except that we report a Gly residue in the N-terminal leader sequence at position −7 whereas MacNeil *et al.* (29) report an Arg at this position.

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